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Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins

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Functional expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in cerebellar granule cells requires stargazin, a member of a large family of four-pass transmembrane proteins. Here, we define a family of transmembrane AMPA receptor regulatory proteins (TARPs), which comprise stargazin, γ-3, γ-4, and γ-8, but not related proteins, that mediate surface expression of AMPA receptors. TARPs exhibit discrete and complementary patterns of expression in both neurons and glia in the developing and mature central nervous system. In brain regions that express multiple isoforms, such as cerebral cortex, TARP–AMPA receptor complexes are strictly segregated, suggesting distinct roles for TARP isoforms. TARPs interact with AMPA receptors at the postsynaptic density, and surface expression of mature AMPA receptors requires a TARP. These studies indicate a general role for TARPs in controlling synaptic AMPA receptors throughout the central nervous system.

Introduction

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)*-type glutamate receptors mediate most fast excitatory synaptic transmission in the brain. These receptors are heterotetramers containing at least two distinct glutamate receptor (GluR) subunits (GluR1–4). Interestingly, AMPA receptors recycle rapidly at the plasma membrane, and activity-dependent changes in synaptic AMPA receptor number regulate synaptic strength. This critical role for glutamate receptor trafficking in synaptic function and plasticity has motivated intensive study of proteins involved in receptor clustering at the postsynaptic density (PSD).

A major breakthrough in understanding synaptic organization was the discovery that proteins containing postsynaptic density-95, discs large, zonula occludens (PDZ) domains are abundant at the PSD and play key roles in recruiting receptors (Kornau et al., 1997; Craven and Bredt, 1998; Garner et al., 2000; Sheng and Sala, 2001). PDZ domains are modular protein–protein interaction motifs that bind to short peptide sequences that often occur at the COOH termini of their protein ligands. The prototypical PDZ protein, PSD-95, a membrane-associated guanylate kinase, was first found to interact with the COOH termini of *N*-methyl-D-aspartate (NMDA) receptor 2 subunits and certain K^+ channels. More recent studies have shown that PICK1 (Xia et al., 1999) and GRIP/ABP, other synaptic PDZ proteins (Dong et al., 1997; Srivastava et al., 1998), bind directly to the tail of AMPA receptor subunit GluR2 and regulate receptor clustering and/or retention at the PSD (Osten et al., 2000). PDZ domains from SAP-97, a PSD-95–related protein, bind the tail of GluR1, but this appears not to regulate synaptic AMPA receptors (Sans et al., 2001; Klocker et al., 2002).

The first transmembrane protein found to interact with AMPA receptors is the tetraspanning protein stargazin (Chen et al., 2000), which is mutated in epileptic stargazer mice (Letts et al., 1998). In addition to absence epilepsy, stargazer mice show cerebellar ataxia (Noebels et al., 1990). Cellular and physiological studies of these mutant mice show that stargazin is required for surface expression of

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*Abbreviations used in this paper: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DAB, 3′,3-diaminobenzidine tetrahydrochloride; EndoH, endoglycosidase H; GluR, glutamate receptor; NMDA, *N*-methyl-D-aspartate; PDZ, postsynaptic density-95, discs large, zonula occludens; PMP, peripheral myelin protein; PSD, postsynaptic density; TARP, transmembrane AMPA receptor regulatory protein.

Key words: synapse; glutamate receptor; plasticity; stargazin; trafficking

AMPA receptors in cerebellar granule cells, whereas AMPA receptors in many forebrain neurons are intact (Chen et al., 1999; Hashimoto et al., 1999). Expression of AMPA receptor protein subunits GluR2 and GluR4 in stargazer granule cells is largely maintained, but these receptors are not delivered to the cell surface. This AMPA receptor deficiency is preserved in cultured granule cells from stargazer, suggesting it is a cell autonomous defect (Chen et al., 2000). Transfecting these cultures with stargazin restores the normal distribution of AMPA receptor function.

Stargazin also has a COOH-terminal PDZ-binding site that associates with PSD-95 (Chen et al., 2000). Importantly, transfecting stargazer granule cells with a stargazin mutant lacking the PDZ-binding site restores extrasynaptic but not synaptic AMPA receptors. This suggests two separable roles for stargazin effects on AMPA receptors. First, stargazin is essential for the delivery and/or maintenance of plasma membrane AMPA receptors. Second, PDZ domain interactions with stargazin mediate synaptic clustering of AMPA receptors. In support of this two-step model, overexpression of stargazin in hippocampal slice cultures selectively augments the number of extrasynaptic AMPA receptors (Schnell et al., 2002). Clustering of these additional receptors to the synapse requires increased levels of the synaptic stargazin anchor, PSD-95 (Schnell et al., 2002). Interestingly, the PDZ-binding site of stargazin contains a consensus site for numerous neuronal protein kinases, and phosphorylation of this site disrupts interaction with PSD-95 (Choi et al., 2002) and prevents synaptic clustering of AMPA receptors (Chetkovich et al., 2002). Although the PDZ-binding site of stargazin serves as a protein kinase A substrate in vitro, it is not clear which kinases phosphorylate the site in neurons.

Stargazin protein has four transmembrane domains and shares some sequence homology with γ-1, an auxiliary subunit of skeletal muscle calcium channels (Letts et al., 1998). In cell transfection studies, stargazin modulates the functional properties of neuronal calcium channels (Letts et al., 1998; Klugbauer et al., 2000; Green et al., 2001; Rousset et al., 2001), and aberrant calcium channel regulation may explain the spike-wave seizure phenotype of stargazer mice (Zhang et al., 2002). In addition to γ-1 and stargazin/γ-2, six additional γ subunit–related genes have been identified, and some of these can apparently also modulate calcium channel function (Klugbauer et al., 2000; Burgess et al., 2001; Chu et al., 2001; Moss et al., 2002). The relationship between calcium channel and AMPA receptor regulation by stargazin and other γ subunits is unclear. This dual role for stargazin and relatives is especially intriguing because AMPA receptors function at the PSD, whereas calcium channels are highly expressed at nerve terminals compared with the PSD.

In this study, we define a family of stargazin-related proteins, γ-3, γ-4, and γ-8, that regulate AMPA receptors. This group of four proteins, in contrast to other calcium channel γ subunits and other related four-pass transmembrane proteins, promote surface expression of functional AMPA receptors. Therefore, we refer to this subset as the family of transmembrane AMPA receptor regulatory proteins (TARPs). TARP family members each show specific complementary patterns of expression throughout the brain and collectively appear to occur in all neuronal types in adult brain. A specific TARP isoform also associates with AMPA receptors in developing brain and in glial cells. In brain regions that express multiple isoforms, TARP complexes remain strictly segregated, suggesting differential functions for distinct AMPA receptor/TARP combinations. Immunofluorescent and electron microscopic studies show that TARPs occur in dendrites, but not axons or presynaptic terminals, and specifically cluster at the PSD together with AMPA receptors. Cerebellar granule cells from stargazer mice, which lack any TARP expression, show immature glycosylation and diminished surface expression of AMPA receptors, suggesting that the receptors are retained in an intracellular domain. These studies identify a universal role for the family of TARPs in regulating surface expression of mature AMPA receptors throughout the brain.

Results

Functional identification of a family of TARPs

Although stargazer mice lack functional AMPA receptors in cerebellar granule cells, AMPA receptors in forebrain neurons are intact (Chen et al., 1999; Hashimoto et al., 1999). This selective loss of AMPA receptor function in cerebellar granule cells might be explained by the expression of stargazin-related proteins in forebrain (Chen et al., 2000; Klugbauer et al., 2000; Sharp et al., 2001). Stargazin/γ-2 is related to a large family of transmembrane proteins (Tomita et al., 2001) including the following: γ-1, a stoichiometric component of skeletal muscle calcium channels (Jay et al., 1990; Hofmann et al., 1999); neuronal calcium channel γ-3, γ-4, γ-5, γ-6, γ-7, and γ-8 (Klugbauer et al., 2000; Burgess et al., 2001; Moss et al., 2002); claudin family proteins, cell adhesion molecules essential in forming epithelial tight junctions (Morita et al., 1999); and others. To determine whether these relatives share stargazin's AMPA receptor regulatory function, we cloned their cDNAs (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200212116/DC1) and assessed their capacity to rescue AMPA receptor–mediated responses in cerebellar granule cells from stargazer mutant mice. As reported previously (Chen et al., 2000), transfection of GFP-tagged stargazin into these neurons restored glutamate-evoked responses recorded at –80 mV, which are almost exclusively mediated by AMPA receptors (Fig. 1 A). Similarly, transfecting these neurons with GFP-tagged γ-3, γ-4, or γ-8 effectively rescued AMPA receptor currents (Fig. 1 A). On the other hand, transfecting GFP-tagged γ-1 calcium channel subunit, γ-5, or claudin-1 failed to rescue AMPA receptor currents (Fig. 1 A). None of the transfections altered NMDA receptor currents as indicated by similar currents recorded at +60 mV. Furthermore, all γ subunit isoforms and claudin-1 were expressed at similar levels as reflected by their GFP fluorescence (unpublished data).

The four proteins that functionally rescue AMPA receptors in stargazer mice cluster on one branch of a phylogenic tree (Fig. 1 B) and comprise the TARP family. Because γ-5 and claudin-1, the proteins most closely related to the TARPs, fail to rescue (Fig. 1 A), we assume that we have identified the complete functional set.

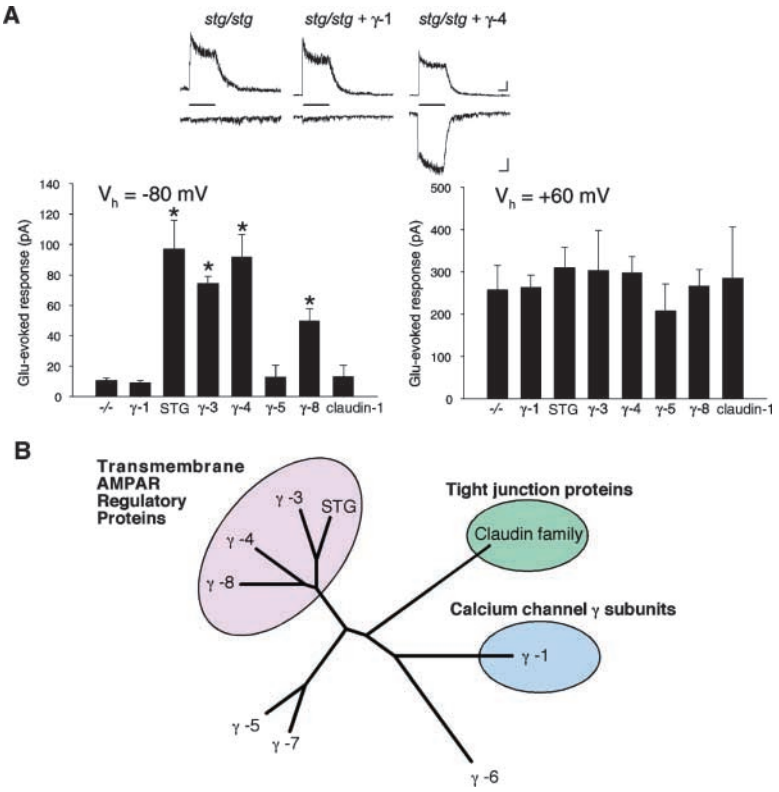

Figure 1. **Functional identification of a family of TRAPs.** (A) Cerebellar granule cells from stargazer mutant mice were transfected with γ-1, stargazin (STG), γ-3, γ-4, γ-5, γ-8, or claudin-1, and whole cell responses to glutamate were recorded. The bar between traces represents 3-s applications of glutamate (100 μM) and cyclothiazide (100 μM). Calibration bars: 50 pA, and 1 s TARP family proteins. stargazin (STG), γ-3, γ-4, and γ-8 restore AMPA-type glutamate-evoked responses (recorded at –80 mV) in *stg/stg* cerebellar granule cells ($n = 5$, $P < 0.01$), but γ-1, γ-5, and claudin-1 do not. The NMDA-type glutamate-evoked responses (recorded at +60 mV) were not significantly altered by any of the transfections. Bottom bar graphs summarize data from these neuronal transfection experiments. (B) Phylogenetic tree shows relationship of TARPs to other related four pass transmembrane proteins.

Cellular distribution of TARPs in brain

To assess the regional distribution of TARPs in brain, we generated and affinity purified antipeptide antibodies specific for each isoform. Western blotting on protein extracts from cerebellum, cerebral cortex, hippocampus, and olfactory bulb showed that each TARP was enriched in a different brain region. Highest levels of γ-2 occur in cerebellum, γ-3 in cerebral cortex, γ-4 in olfactory bulb, and γ-8 in hippocampus (Fig. 2 A). To determine the cellular distribution of the TARPs, we performed in situ hybridization on sagittal and coronal sections of adult rat brain with ^{35}S-labeled antisense probes, which revealed distinctive patterns for each TARP (Fig. 2, B and C). As previously reported, stargazin/γ-2 mRNA is discretely expressed in specific neuronal populations in numerous brain regions and occurs at highest levels in cerebellum but also occurs in cerebral cortex, hippocampus, and facial nerve nucleus. At higher power, images show that stargazin in hippocampus occurs both in pyramidal cells and interneurons (Fig. 3 E), and γ-3, also expressed exclusively in neuronal cells, occurs at highest levels in cerebral cortex (Fig. 2, B and C). By contrast, γ-4 is expressed both in specific neuronal populations, such as the caudate putamen, but also appear in nonneuronal cells as suggested by expression in white matter of the corpus callosum (Fig. 2, B and C).

To evaluate in more detail γ-4 expression in white matter, we examined the in situ hybridization patterns at higher magnification. This revealed high expression of γ-4 in scattered cells in white matter of cerebellum and corpus callosum (Fig. 3, B and F). Immunocytochemical staining revealed a stellate shape for these cells indicative of glial cells, which may also express AMPA receptors (Fig. 3, C, D, G, and H). To confirm that γ-4 occurs in nonneuronal cells, we prepared glial and neuronal cultures from hippocampus. Western blotting showed that γ-4 occurs in both the neuronal and glial cultures, whereas γ-8 is expressed only in the neurons (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200212116/DC1).

The most striking distribution for a TARP is that of γ-8, which is dramatically enriched in hippocampus, both in neurons of the dentate gyrus and pyramidal neurons in Ammon's horn (Fig. 2, B and C). It is also important to note that stargazin but not other TARPs are expressed in cerebellar granule cells (Fig. 2 B), which can explain the selective defect of AMPA receptors in those neurons (Hashimoto et al., 1999).

We also explored the developmental course of TARP expression during rat ontogeny. Western blotting of brain cerebral cortex homogenates shows that γ-2, γ-3, and γ-8 are expressed at low levels in newborn and neonatal brain and increase to reach highest levels in adult (Fig. 4 A). By contrast, γ-4 expression peaks at postnatal day 6 and decreases through later development. In situ hybridization confirmed the robust expression of γ-4 but not other TARPs in E16 embryo (Fig. 4 B). High levels of γ-4 expression occur in neurons throughout the developing central and peripheral nervous systems. Some expression of γ-4 is found also in nonneuronal cells, such as epithelial cells lining the intestines (Fig. 4 B), but γ-4 is absent from most peripheral tissues.

Clustering of TARPs with AMPA receptors at the postsynaptic density

To determine whether TARPs in neurons colocalize with AMPA receptors at synapses, we developed a pan-TARP antibody raised to the conserved cytosolic tail of stargazin; this

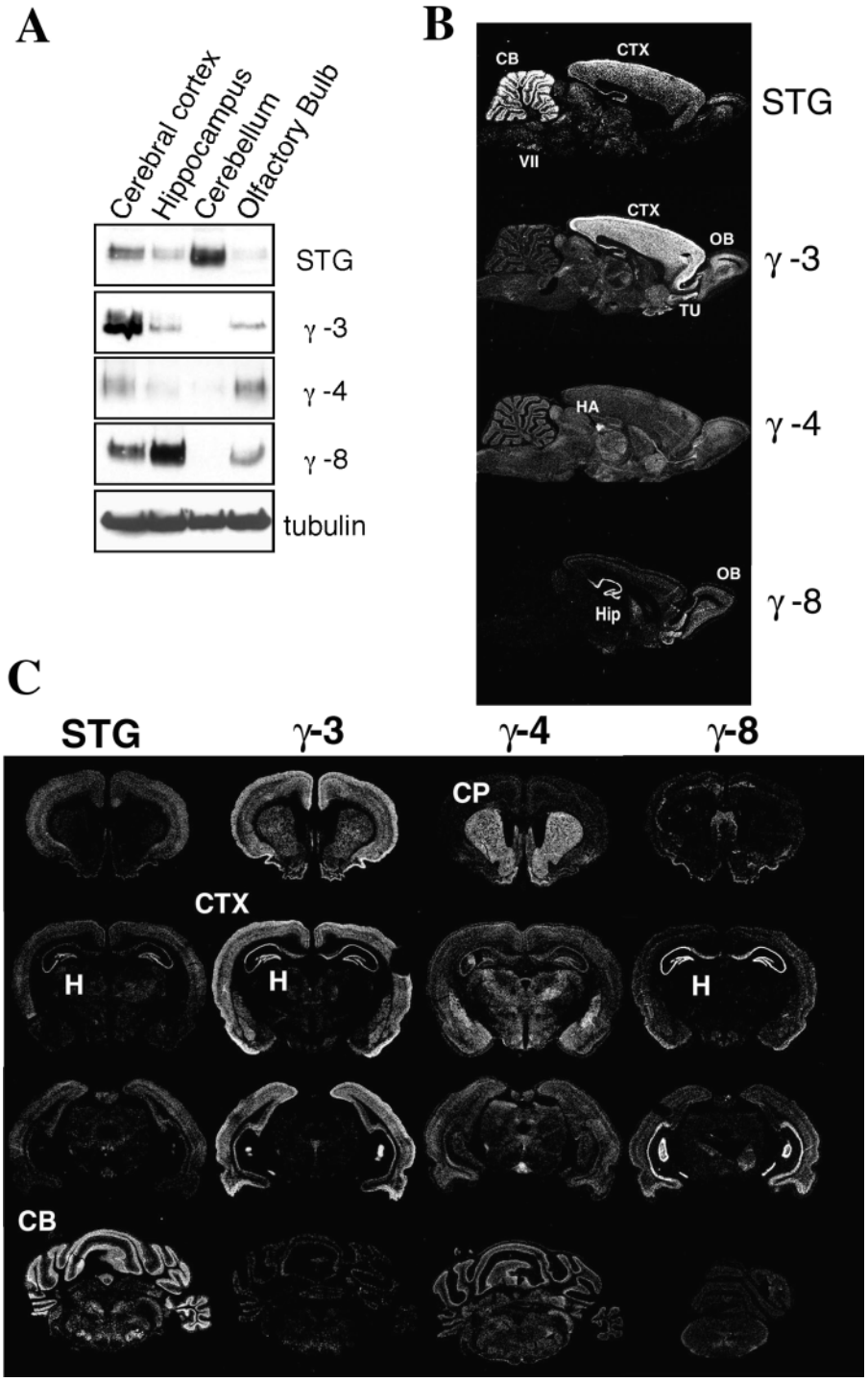

Figure 2. **Differential distribution of TRAPs in adult brain.** (A) Immunoblotting shows highest levels of stargazin (γ-2) in cerebellum, γ-3 in cerebral cortex, γ-4 in olfactory bulb, and γ-8 in hippocampus. In situ hybridization on sagittal (B) and coronal (C) sections show that stargazin mRNA is concentrated in cerebellum and also occurs in cerebral cortex (CTX), hippocampus (H), and facial nerve nucleus (VII). γ-3 is restricted to forebrain and occurs at highest levels in cortex, hippocampus, and olfactory tubercle (TU) and at lower levels in caudate putamen. γ-4 occurs diffusely throughout the adult brain but appears distinctly enriched in caudate putamen (CP) and habenula (HA). γ-8 mRNA is highly enriched in hippocampus and modestly expressed in cortex and olfactory bulb (OB).

antibody reacts with all four TARPs (unpublished data). Immunofluorescent labeling of primary neuronal cultures showed that TARP immunoreactivity occurs selectively at punctate sites along the dendrites (Fig. 5). These puncta reflect excitatory synapses as they overlap with AMPA receptor subunit GluR2 and with PSD-95 (Fig. 5) but not with the GABA synthesizing enzyme GAD-65 (Fig. 5). Not all excitatory (NMDA receptor–positive) synapses in cultured neurons express AMPA receptors (Gomperts et al., 1998; Liao et al., 1999), and these reflect the "silent synapses" (Isaac et al., 1995; Liao et al., 1995). Indeed, we found that TARP colocalizes with only 53 ± 16% of synapses labeled for NR1, whereas TARP colocalizes with >84 ± 6% of punctate sites labeled for GluR2 (Fig. 5).

Subcellular fractionation studies showed that all four TARPs are enriched in synaptosomes and are resistant to extraction with 0.5% Triton X-100, suggesting association with the PSD (Fig. 6 A). To determine decisively whether TARPs localize to the PSD, we used immuno-EM labeling in adult hippocampus (Fig. 6). Preembedding immunocytochemistry using a pan-TARP antibody and 3′,3-diaminobenzidine tetrahydrochloride (DAB) produced dense label-

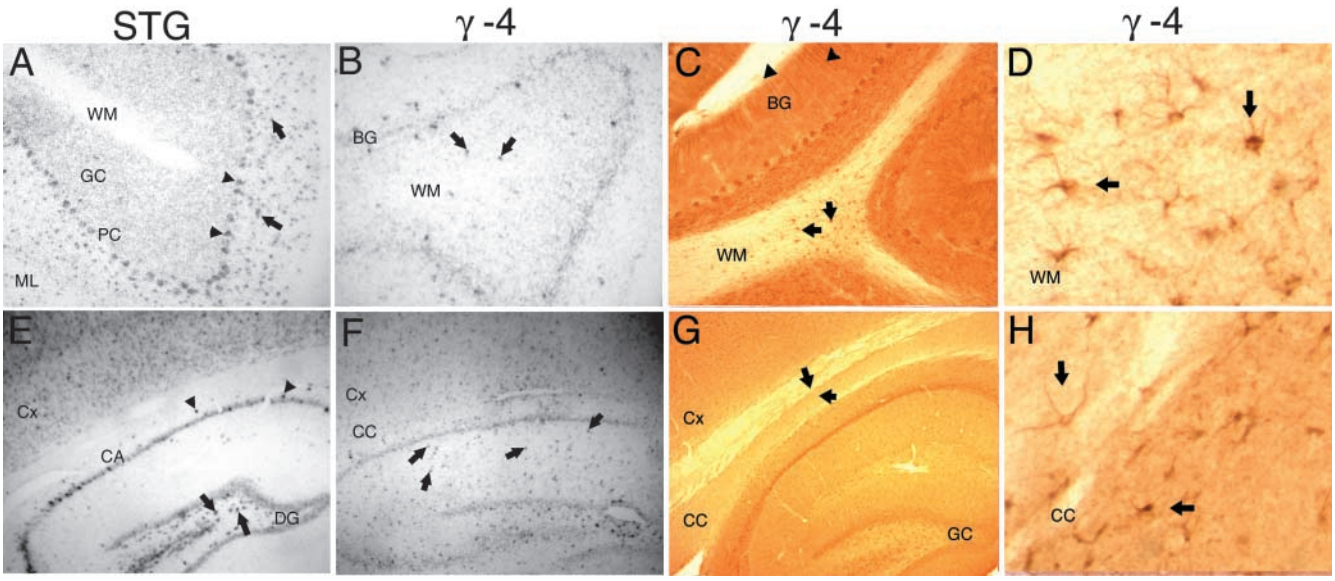

Figure 3. **γ-4 is expressed in glial cells in adult rat.** In situ hybridization (A, B, E, and F) and immunocytochemistry (C, D, G, and H) show that γ-4 but not stargazin (STG) is expressed in glial cells. (A) In cerebellum, stargazin mRNA is expressed in granule cells (GC), Purkinje cells (PC; arrowheads), and in interneurons (arrows) in molecular layer (ML). (B) In contrast, γ-4 mRNA occurs in a diffuse band along the Purkinje cell layer that reflects expression in Bergmann glia (BG) as indicated in C by immunostaining of their apical processes in the molecular layer (arrows). Scattered cells in cerebellum showing γ-4 mRNA expression (B, arrows) are glia, since immunostaining shows their fibrous star-shaped morphology (D, arrows). (E) In neocortex, stargazin mRNA is expressed in neurons of cerebral cortex (Cx), granule cells of dentate gyrus (DG), and pyramidal cells of the hippocampus (CA). Stargazin is also expressed in interneurons of hilus of dentate gyrus (arrows) and in stratum oriens (arrowheads) and stratum radiatum. (F) In contrast, γ-4 mRNA occurs in scattered cells throughout cerebral cortex (Cx), corpus callosum (CC), and hippocampus (arrows) that resemble glia as detected by immunocytochemistry (G and H).

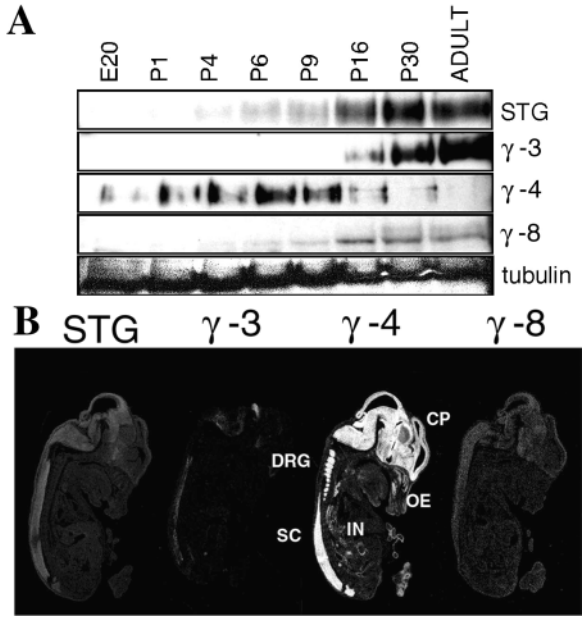

Figure 4. **Developmental switch in neuronal expression of TARPs.** Immunoblotting shows that γ-4 protein levels peak in rat pups at postnatal day 6 (P6) and decline thereafter in cerebral cortex. In contrast, expression of stargazin (STG), γ-3, and γ-8 appear later and progressively increase during animal maturation. In situ hybridization at embryonic day 16 (E16) shows that γ-4 mRNA but not that of other TARPs occurs at extremely high levels throughout the nervous system in cortical plate (CP), olfactory epithelium (OE), dorsal root ganglia (DRG), and spinal cord (SC). Some γ-4 mRNA also occurs in peripheral tissues such as the intestine (IN).

ing in the hippocampus (control sections were unlabeled). At the EM level, the neuropil showed dense labeling restricted almost entirely to the dendrites and postsynaptic spines (Fig. 6 B). Densest labeling was seen in some patches in the dendrites and in the postsynaptic densities. We used immunogold electron microscopic studies to assess possible colocalization of TARPs with AMPA receptors at the ultrastructural level (Fig. 6, C–L). Double labeling of hippocampal sections with antibodies to GluR2/3 (large particles) and a pan-TARP antibody (small particles) showed that both are concentrated on the postsynaptic side of excitatory synapses (Fig. 6, C–H and L). In addition, close association of gold particles corresponding to GluR2/3 and TARP-like proteins were also found, though less frequently, in dendritic cytoplasm (Fig. 6, I–K).

We next performed immunoprecipitation experiments to determine whether TARPs associate with AMPA receptors in neurons. Cerebellar membranes were solubilized with Triton X-100 and were immunoprecipitated with an antibody to stargazin. AMPA receptor subunits GluR1, GluR2, and GluR4 coimmunoprecipitated with stargazin in extracts from wild-type (unpublished data) or heterozygous mice but not from stargazer mice (Fig. 7 A). Immunoprecipitation studies indicated that AMPA receptors also associate with the other TARP isoforms in brain tissues expressing high TARP levels. Thus, AMPA receptors coprecipitate with γ-3 in cerebral cortex, with γ-8 in hippocampus and with γ-4 in neonatal forebrain (Fig. 7, B–D). As controls, we found that K^+ channel Kv 1.4, $GABA_A$ receptor subunit-β and neuronal cadherin did not associate with TARPs.

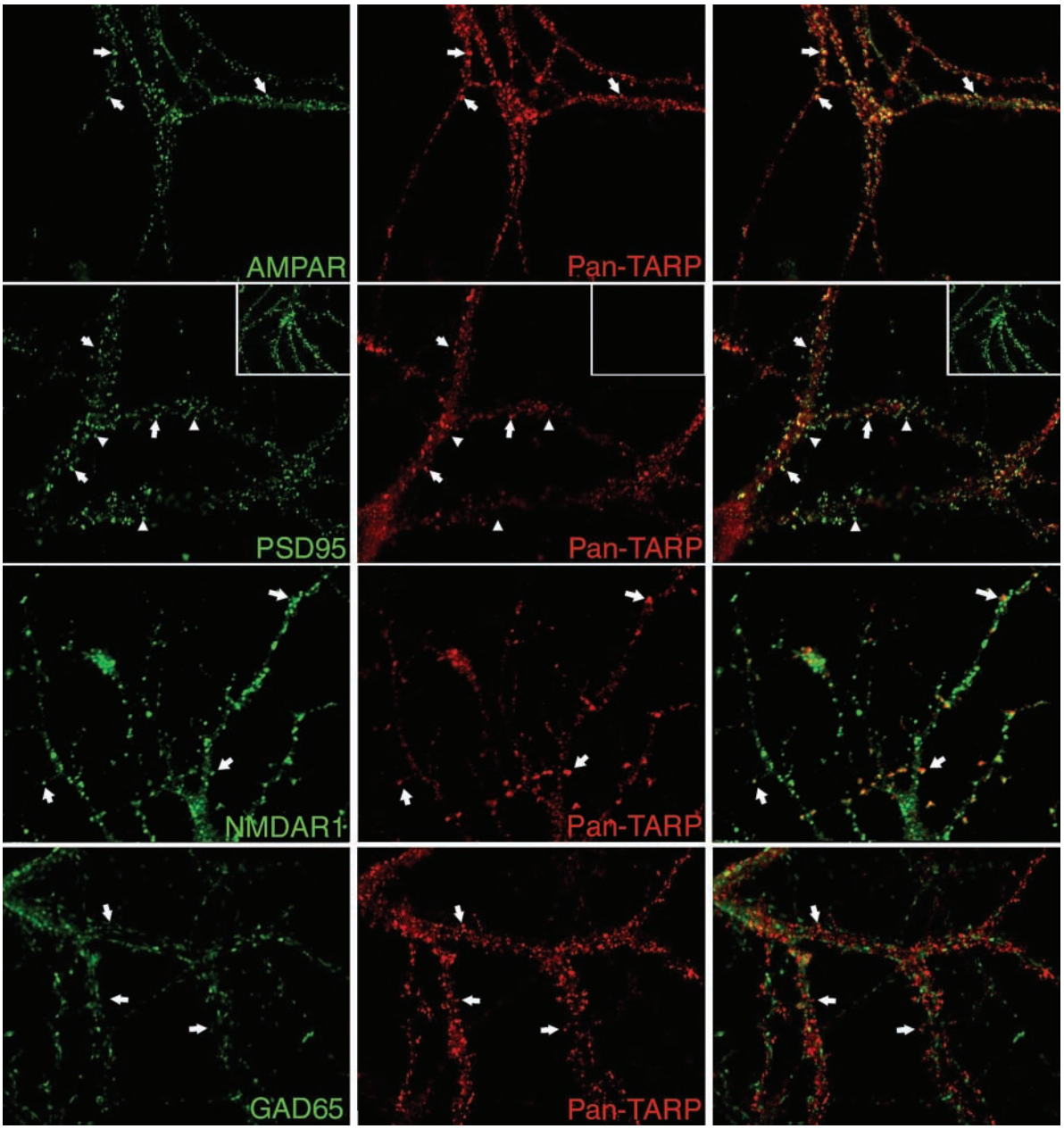

Figure 5. **TARPs cluster selectively at excitatory synapses that contain AMPA receptors.** An antibody (pan-TARP) that reacts with all TARPs was used to stain cultured hippocampal cultures. (First row) Immunofluorescence for TARPs occurs at punctate sites (arrows) along the dendrites that closely colocalize with the GluR2 subunit of the AMPA receptor (AMPAR). (Second row) Virtually all TARP puncta colocalize with PSD-95 (arrows), but some PSD-95 puncta lack TARP immunofluorescence (arrowheads). (Third row) Almost all TARP puncta colocalize with NMDAR1 (arrows), but some NMDAR1 puncta lack TARP immunofluorescence. (Fourth row) TARPs show no overlap with the GABAergic marker GAD65. Preabsorbing the antibody with antigen (10 μM) blocks labeling (small boxes in second row).

TARPs appear to interact with multiple AMPA receptor subtypes. Although γ-4 in neonatal cortex associates with AMPA receptors containing GluR2/3–GluR4, γ-4 in adult cerebellum, which is expressed in Bergmann glia, associates with GluR1 and GluR4 (Fig. 7 E). Despite this promiscuity of TARP interaction with multiple AMPA receptor subtypes, TARP–AMPA receptor complexes apparently do not intermix. Although cerebrocortical pyramidal neurons express multiple if not all TARP isoforms (Fig. 2), immunoprecipitation shows that they are strictly segregated such that TARPs do not form heteromeric complexes (Fig. 7 F).

To determine whether the interaction of TARPs with AMPA receptors occurs on the cell surface, we treated cultured cortical neurons with a cell-impermeable protein cross-linker, then solubilized the cells with SDS. Immunoprecipitation analysis showed that AMPA receptor subunit GluR2 but not a control synaptic transmembrane protein, N-cadherin, coimmunoprecipitated with γ-3 in a cross-linker–dependent fashion (Fig. 7 G). Finally, we assessed association with AMPA receptors at the PSD. Because the interaction of TARP with AMPA receptors is sensitive to the harsh detergents needed to solubilize the PSD (Chen et al., 2000), we treated partially purified PSD fractions

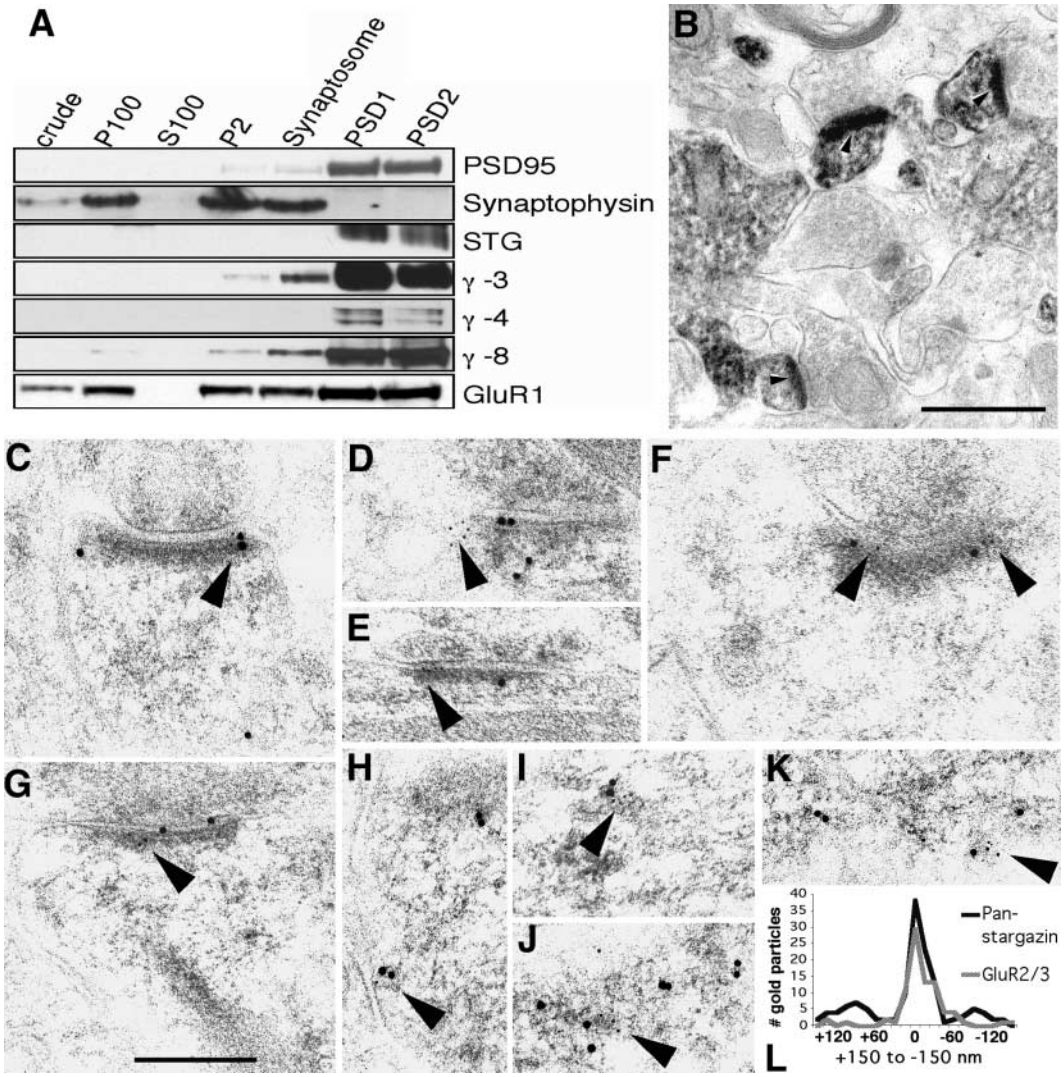

Figure 6. **TARPs colocalize with AMPA receptors at excitatory synapses.** (A) Western blotting shows that AMPA receptor trafficking proteins are all highly enriched in PSD fractions. By contrast, synaptophysin, which is also enriched in the crude synaptosomes is extracted with 0.5% Triton X-100 and does not occur in PSD fractions. (B) Pre-embedding DAB immunoperoxidase labeling for pan-TARP in the CA1 stratum radiatum of the hippocampus. Labeling is concentrated in dendritic shafts and associated postsynaptic spines. Note the dense concentration of labeling at postsynaptic densities (arrowheads). Patches of labeling also are evident in the spine and dendrite cytoplasm. (C–L) Double immunogold labeling for pan-TARP (5 nm gold) and GluR2/3 (10 nm gold) in the hippocampus. (C, D, and H) CA1 stratum radiatum. (F and G) Hilus. (E and I) CA3 stratum lucidum. (J) Molecular layer of the dentate gyrus. (K) CA1 stratum oriens. Black arrowheads indicate 5-nm gold particles that is associated closely with 10 nm gold particles. In C and I, one of the 5-nm gold particles overlaps a 10-nm gold particle. Close associations of 5- and 10-nm gold particles are more common at synapses (C–H) than in dendrite cytoplasm (I–K). H shows a very oblique synapse; 5 and 10-nm gold particles are associated in a presumptive vesicle fused to the lateral wall of the postsynaptic spine. Bars: (B) 500 nm; (in G, for C–K) 200 nm. (L) Distribution of gold particles (y axis) in the perpendicular (axodendritic) axis of synapses. Zero represents the postsynaptic membrane; + is toward presynaptic and – is toward postsynaptic. Note how most gold for TARPs and GluR2/3 is concentrated at the postsynaptic membrane and within the postsynaptic density.

from cerebral cortex with a protein cross-linker before solubilization and immunoprecipitation in the harsh detergent condition. These experiments (Fig. 7 H) showed that γ-3 does indeed associate at the PSD with GluR2 but not with CaMKII.

Interaction with TARPs promotes surface expression of mature AMPA receptors

To determine directly whether stargazin is essential for AMPA receptor trafficking to the plasma membrane, we quantified the surface expression of glutamate receptor subunits in stargazer cerebellar granule cells, which lack expression of any TARP isoform. Cells were treated with a membrane-impermeable biotin reagent, and surface receptors were captured on avidin-linked agarose. These experiments revealed an ~75% decrease in the surface expression of AMPA receptor subunit GluR2 in cerebellar cells from stargazer (Fig. 8 A), whereas total GluR2 levels were decreased by only 10–20% in stargazer cerebellum. As these cultures are a mixture of neurons, the remaining surface GluR2 in stargazer may represent receptors in Purkinje cells, which are enriched in GluR2 (Hampson et al., 1992; Lambolez et al., 1992) and are unaffected in stargazer mice (Chen et al.,

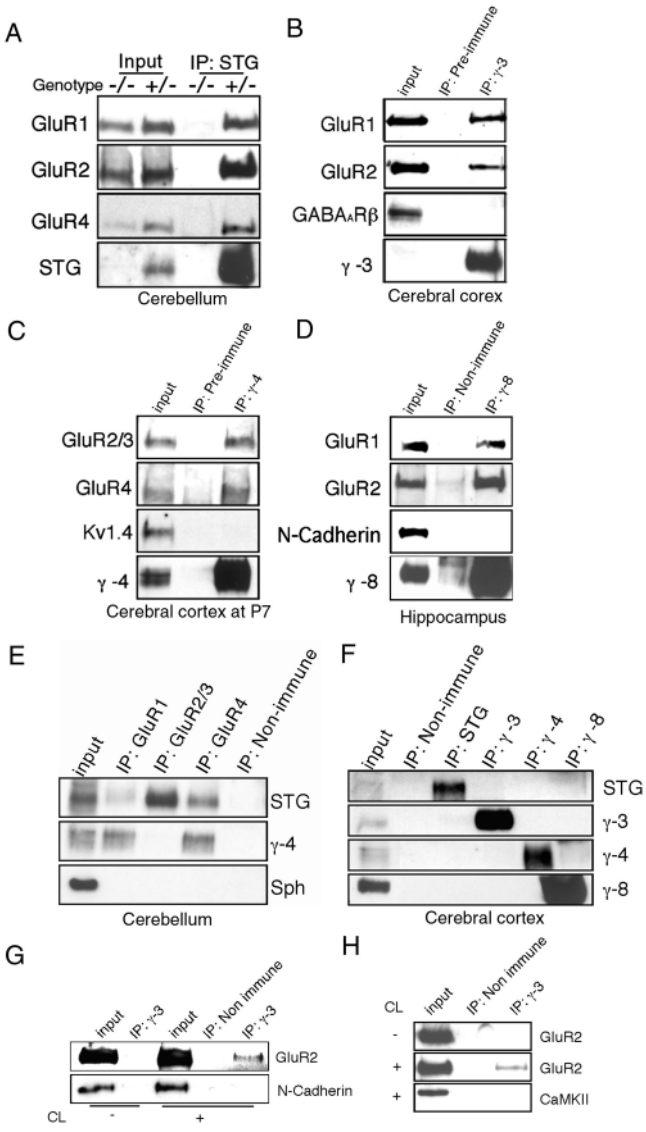

Figure 7. **TARPs interact specifically with AMPA receptors in brain extracts.** (A) AMPA receptor subunits GluR1, GluR2, and GluR4 coimmunoprecipitate with stargazin (STG) in brain extracts from *+/stg* mice (+/−). In extracts from *stg/stg* mice (−/−), the antistargazin antibody does not immunoprecipitate GluR1, GluR2, or GluR4. (B–D) AMPA receptor subunits also coimmunoprecipitate with γ-3, γ-4, and γ-8 in extracts from adult cerebral cortex, postnatal day 7 cerebral cortex, and hippocampus, respectively. As control, other transmembrane proteins such as $GABA_AR\beta$, N-cadherin, and Kv1.4 did not coimmunoprecipitate. (E) Subunit specificity for TARP interactions in cerebellum. Stargazin (STG) and γ-4 coimmunoprecipitate with GluR1 and GluR4, but only stargazin coimmunoprecipitates with GluR2/3 in cerebellum. (F) TARP isoforms are strictly segregated, since they do not coimmunoprecipitate with one another in cerebral cortex. (G) Surface GluR2 coimmunoprecipitates with γ-3. Primary cerebrocortical cultures were incubated with a membrane impermeable cross-linker (CL), and after SDS solubilization extracts were immunoprecipitated for γ-3; GluR2 coprecipitates with γ-3 in a cross-linker–dependent fashion, whereas another synaptic transmembrane protein, N-cadherin, does not. H, GluR2 coimmunoprecipitated with γ-3 in chemical cross-linked PSD fractions from brain, whereas another PSD protein, CaMKII, does not.

1999; Hashimoto et al., 1999). This decrease in surface expression is selective for AMPA receptors, since NMDAR2A was unaffected (Fig. 8 A).

Control of receptor expression at the plasma membrane is often regulated by quality control mechanisms for exit from the ER (Rothman, 1987; Hurtley and Helenius, 1989). Because granule cells are small and their soma are largely occupied by the nucleus, it is difficult to visualize their ER by light microscopy. As an alternative, we took advantage of the processing of glutamate receptors by *N*-glycosylation. That is, glutamate receptors receive high mannose glycosylation in the ER and are modified with more complex sugars in the Golgi (Hollmann et al., 1994; Sans et al., 2001). We distinguished these glycosylation patterns with endoglycosidase H (EndoH), which only digests the immature high mannose sugars, and PNGaseF, which removes all N-linked carbohydrates. These experiments showed that 40–50% of the GluR2 in cerebellum of stargazer mice is sensitive to EndoH (Fig. 8 B), likely reflecting an immature pool in the ER. The cleaved GluR2 band in the EndoH-treated stargazer cerebellum comigrated with the completely deglycosylated subunit obtained by treating with PNGaseF (Fig. 8 B). By contrast, GluR2 is almost entirely resistant to EndoH in the cerebellum of heterozygous mice (Fig. 8 B) as has been described previously for wild-type mice (Sans et al., 2001). The small shift of GluR2 in wild-type and heterozygous mice is explained by differential processing of multiple glycosylation

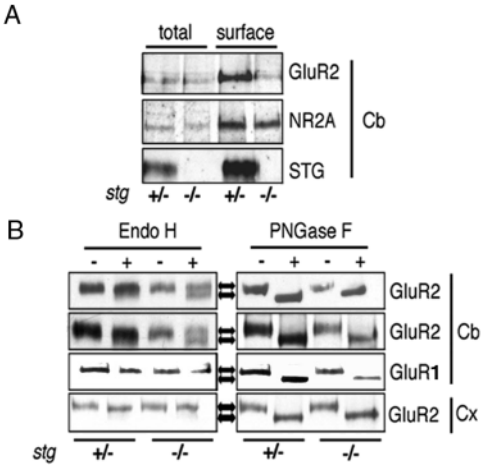

Figure 8. **Stargazin enhances mature glycosylation and surface expression of an AMPA receptor subunit protein.** (A) Cerebellar (Cb) cultures from *+/stg(+/−)* or *stg/stg(−/−)* were biotinylated, solubilized, and precipitated with streptavidin-agarose. Surface expression of the AMPA receptor subunit GluR2 is dramatically decreased in *stg/stg* cultures, whereas NMDAR subunits NR2A are not significantly altered. (B) In cerebellum of stargazer mouse (−/−), a large fraction of GluR2 remains immature and sensitive to EndoH glycosidase. By contrast, GluR2 in stargazer cerebral cortex (Cx) is mature and resistant to EndoH. In all cases, GluR2 glycosylation is removed by the nonspecific N-glycosidase, PNGaseF. Total levels of GluR2 were consistently decreased by ∼10–20% in cerebellum from *stg/stg* mice. Note that two independent examples from stargazer cerebellum are presented.

sites (Standley et al., 1998; Sans et al., 2001). As a control, we found that GluR1, which is not expressed in cerebellar granule cells and binds to γ-4 in cerebellum (Fig. 7 E), showed normal resistance to EndoH in stargazer cerebellum (Fig. 8 B). In cerebral cortex, GluR2 is EndoH resistant in both heterozygous and homozygous stargazer mice (Fig. 8 B; consistent with the cerebellar-specific defect of functional AMPA receptors in stargazer (Chen et al., 1999; Hashimoto et al., 1999). Treatment with PNGaseF digests GluR2 equivalently in all cases (Fig. 8 B).

Discussion

This study demonstrates that stargazin (γ-2) is the prototypical member of a family of four TARPs, including γ-3, γ-4, and γ-8, that can promote expression of functional AMPA receptors at the neuronal plasma membrane. Members of the TARP family all associate independently with AMPA receptors and cocluster with the receptors at postsynaptic sites. Complementary expression patterns for TARP family proteins in the central nervous system appear collectively to cover all populations of neurons and glia that express AMPA receptors, suggesting a general role for this regulatory mechanism.

The TARPs are related to a larger superfamily of junctional transmembrane proteins

TARPs are members of a large superfamily of four pass transmembrane receptors that include the γ-1 subunit of the skeletal muscle calcium channel, claudin family tight junction proteins, peripheral myelin protein (PMP)22 and others. Proteins in this superfamily are all enriched at sites of cell–cell contact but subserve diverse functions in distinct cell types throughout the body. Genetic analyses have identified a major role for this superfamily in human diseases. Deletion or duplication of PMP22 cause peripheral neuropathies (Warner et al., 1999), mutations in claudin-16 cause hypomagnesemia (Simon et al., 1999), and mutations in claudin-14 cause deafness (Wilcox et al., 2001).

Sequence comparison shows that TARPs form a small branch on this larger phylogenetic tree, and our data suggest that the four TARP members are the only proteins of this type that can regulate AMPA receptors. Proteins in the TARP family share 60% sequence similarity to each other, 23% to claudin-1, 23% to γ-1 calcium channel subunit, and only 21% to PMP22. The specific domains or sequences within TARPs that specify their interactions with and regulation of AMPA receptors are uncertain. Future studies constructing chimeras between TARPs and their closest functionally inactive relatives should help identify these critical regions.

TARP family members are dubbed γ subunits of calcium channels and share homology to the γ-1 subunit, which was purified as a stoichiometric component of skeletal muscle calcium channels (Jay et al., 1990; Hofmann et al., 1999). γ-1 influences on calcium channels include effects on voltage dependence, current amplitude, and current kinetics but these results are controversial. Stargazin/γ-2 and γ-3 also appear to interact with neuronal calcium channels (Kang et al., 2001). The functional roles for these interactions are complex and depend on the specific calcium channel subunits studied and the cation used as charge carrier (Ba^{2+} versus Ca^{2+}) (Rousset et al., 2001). However, most studies show that stargazin causes a small but significant hyperpolarizing shift in steady-state inactivation (Letts et al., 1998; Klugbauer et al., 2000; Green et al., 2001; Rousset et al., 2001). In stargazer (γ-2 mutant) mice, the voltage dependence and steady-state inactivation of calcium channel activation is unaltered in cerebellar granule cells (Chen et al., 2000). However, a recent study shows that both high and low voltage–activated calcium channel currents are enhanced in thalamic relay neurons of stargazer (Zhang et al., 2002).

A calcium channel role for stargazin has also been suggested to underlie the epileptic phenotype of stargazer mice, since mutations in calcium channel subunits underlie several spike-wave epilepsies in mice and humans (Zhang et al., 2002). On the other hand, we report here stargazin expression in inhibitory interneurons in forebrain. Therefore, decreased AMPA receptor function on interneurons causing disinhibition of relay neurons may explain stargazer epilepsy. However, we have not yet demonstrated abnormal expression or function of AMPA receptors in interneurons of stargazer mice. Clarifying how stargazin deficiency causes epilepsy and how it can regulate both calcium channels and AMPA receptors remain important issues.

TARPs promote surface expression of mature AMPA receptors

The immature glycosylation of GluR2 we find in granule cells from stargazer cerebellum suggests that stargazin plays a chaperoning role during receptor biogenesis and/or may stabilize the mature receptor. The assembly of several multiprotein receptor complexes, including immune (Klausner et al., 1990), acetylcholine (Blount et al., 1990), and $GABA_B$ receptors (Margeta-Mitrovic et al., 2000), is a tightly controlled process that ensures expression of the correct number of properly composed receptors on the cell surface. The EndoH sensitivity of GluR2 in the stargazer mice suggests that stargazin may perform this quality control function first at the level of the ER/Golgi, since recent studies show that EndoH-sensitive GluR subunits reside in ER microsomes (Greger et al., 2002). Consistent with an interaction of stargazin and AMPA receptors before reaching the PSD, we find that a subpopulation of these proteins colocalizes in the dendritic cytoplasm.

Highest densities of TARP family proteins are found at postsynaptic sites and therefore more closely resemble the distribution of AMPA receptors than that of calcium channels. In cultured hippocampal neurons, TARPs are highly clustered at synapses and colocalize with AMPA receptors. TARPs occur only at excitatory synapses and in this way are more selective for AMPA receptors than are other AMPA receptor–binding proteins such as GRIP/ABP and PICK1, which also occur at GABAergic synapses (Dong et al., 1999; Wyszynski et al., 1999) and dopaminergic synapses, respectively. Cortical pyramidal neurons also have so called "silent synapses" characterized by postsynaptic sites containing PSD-95 and NMDA receptors but lacking AMPA receptors (Gomperts, 1996; Liao et al., 1999). That TARPs precisely colocalize with AMPA receptors and are absent from excita-

tory (PSD-95–positive) synapses lacking AMPA receptors (Fig. 5) suggests a fundamental role for TARPs in controlling synaptic AMPA receptor localization. At the EM level, TARPs are quantitatively enriched in the PSD together with AMPA receptor subunits. We find no evidence for expression of TARPs in axons or presynaptic sites in hippocampus. Although claudin proteins mediate homophilic interactions at tight junctions (Morita et al., 1999), the lack of TARPs in nerve terminals suggests strongly that they do not mediate homophilic adhesion at synapses.

Differential TARP expression and synapse-specific modulation of AMPA receptors

Why might distinct neuronal populations express specific TARP isoforms? One possibility could be that TARP isoforms preferentially associate with specific AMPA receptor subtypes. In certain brain regions, we find that individual TARP isoforms associate with specific GluR subunit combinations. However, such selectivity does not hold in all brain regions, and no one-to-one correspondence between AMPA receptor subtypes and specific TARPs occurs universally. Some neurons, such as cerebrocortical and hippocampal pyramidal cells, express more than one TARP isoform. However, our immunoprecipitation studies show that TARP isoforms do not intermix. This suggests that AMPA receptor complexes contain only one TARP isoform or that TARP–AMPA receptor complexes are strictly segregated. The oligomeric structure of TARP–AMPA receptor complexes and how they are segregated in neurons expressing multiple isoforms remains uncertain.

By forming these distinct complexes with AMPA receptors, TARPs may participate in differential regulation of AMPA receptor turnover at synapses. Activity-dependent synaptic plasticity is often associated with changes in the number of synaptic AMPA receptors (Malenka and Nicoll, 1999; Malinow et al., 2000). Interestingly, physiological studies reveal that AMPA receptor trafficking is controlled in a synapse-specific fashion in distinct brain regions. For example, the endocytosis of AMPA receptors that underlies long term depression at cerebellar Purkinje cell synapses is controlled by PKC (Xia et al., 2000), whereas phosphatase activity underlies NMDA receptor–dependent long term depression at CA1 synapses in hippocampus (Mulkey et al., 1994). Although similar GluR subunit combinations occur in Purkinje cells and CA1 pyramidal cells, the differential expression of TARP isoforms in these cells (predominantly stargazin and γ-3 in Purkinje cells and γ-8 in CA neurons) could explain these differences in regulating AMPA receptor turnover.

How might TARPs contribute to differential regulation of AMPA receptors at specific synapses? The cytoplasmic tails of TARPs all contain a COOH-terminal PDZ-binding site, and synaptic clustering of stargazin requires that this PDZ-binding site interact with PSD-95 (Chen et al., 2000; Schnell et al., 2002). A critical threonine in the PDZ-binding site for the TARPs corresponds to a consensus site for protein phosphorylation, and phosphorylation of this site disrupts stargazin binding to PSD-95 and prevents synaptic expression of AMPA receptors (Chetkovich et al., 2002; Choi et al., 2002). Differential phosphorylation of TARPs at their PDZ-binding sites represents a possible mechanism for synapse-specific regulation. Sequence alignment shows that the family of TARPs shares high sequence identity (54%) in their NH_2-terminal regions that contain the transmembrane and extracellular domains but much lower identity (21%) in their COOH-terminal regions that contain the cytoplasmic tails. This may indicate that the conserved NH_2-terminal region mediates AMPA receptor interaction and regulation and that differential modifications and protein interactions with the divergent cytoplasmic tails would dictate isoform specificity. Targeted disruption of TARP isoforms should help clarify the roles for this family of proteins in regulating AMPA receptors and perhaps other postsynaptic ion channels.

Materials and methods

Antibodies

The following antibodies were used: rabbit polyclonal antibodies to GluR1, GluR2/3, GluR4, and GABAARβ (Chemicon), Kv1.4 (a gift from L. Jan, Howard Hughes Medical Institute, University of California, San Francisco); and mouse monoclonal antibodies to GluR2 (Chemicon), PSD95 (Transduction laboratories), GAD65 (a gift from S. Baekkeskov, Hormone Research Institute, University of California, San Francisco), and N-Cadherin (BD Biosciences). Polyclonal antisera to stargazin family proteins were raised by injecting rabbits or guinea pig with a GST–stargazin fusion protein encoding the last 120 aa of stargazin (pan-TARP) (Chen et al., 2000) or to peptides as follows: stargazin, ClQKDSKDSLHADTANR; γ-3, CQFHNSTPKEFKESLHNNP; γ-4, QMHDFFQQDLKEGFHVSMLC; and γ−8, CASGFLTLHNAFPKEA. Antisera were all affinity purified on agarose columns containing the immunizing antigen.

Immunoprecipitation

For coimmunoprecipitations, P2 postnuclear membranes (Luo et al., 1997) were suspended in lysis buffer containing TEEN (25 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl) and 1% Triton X-100, protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin), 1 mM NaF, and 1 mM Na-orthovanadate, and centrifuged at 37,000 *g* for 40 min. Supernatants were then incubated with 1 μg of affinity-purified antibodies and 20 μl of protein A–sepharose beads (Sigma-Aldrich). Beads were then washed three times with 1% Triton X-100 in TEEN. Bound proteins were eluted by heating the resin in 20 μl of 2× SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting. Input lanes contained 5% of the protein used for immunoprecipitation. For chemical cross-linking, PSD fractions in 10 mM Hepes, pH 7.4, were incubated with or without 200 μM of the cell permeable cross-linking reagent dithiobis(succinimidylpropionate) (DSP; final 200 μM) on ice for 10 min, and reactions were terminated by adding Tris-HCl, pH 7.4 (final 120 mM). Homogenates were then solubilized by SDS and used for immunoprecipitation assays. Cross-linking experiments using primary cerebrocortical cultures were performed according to the supplier's protocols (Pierce Chemical Co.). In brief, cerebrocortical cultures were washed with PBS twice and gradually cooled down to 4°C. Cells were incubated with the cell impermeable cross-linker 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP; final 2 mM), then with glycine (final 50 mM) for 15 min and finally solubilized with RIPA. These solubilized extracts were processed for immunoprecipitation as described above.

Electrophysiology

Whole-cell patch-clamp recording of cerebellar granule cells transfected with TARPs, γ-1, γ-5, or claudin-1 was performed as described (Chen et al., 2000).

Cloning of rat stargazin isoforms

cDNAs encoding rat γ-3, γ-4, γ-5, γ-8, and claudin-1 were cloned by RT-PCR using rat brain total RNA and primers with the following nucleotide sequences: γ-3 forward, ATGAGGATGTGTGACAG; γ-3 reverse, TCAGACGGGCGTGGTGCGTCT; γ-4 forward, ATGGTGCGATGCGACCG; γ-4 reverse, TCACACGGGAGTCGTCCGCCG; γ-5 forward, ATGAGCGCCTGTGGG; γ-5 reverse, TCAGCAGGGAGATGA; claudin-1 forward, ATGGCCAACGCGGGG; claudin-1 reverse, CACGTAGTCTTTCCC; γ-8

forward, TTTGGTGCGAGAAGGG; and γ-8 reverse, CGCCCAGAATGATGTTCC. This partial γ-8 cDNA is fused with another partial γ-8 cDNA cloned by PCR using BAC as a template. Finally, we cloned full-length γ-8 cDNA. The γ-1 cDNA was cloned by RT-PCR using rat skeletal muscle total RNA as template and the following primer pair: forward, ATGTCACAGACCAAA and reverse, CTAGTGCTCTGACTC. These PCR products were subcloned into pGW1 (British Biotechnology), and EGFP was inserted into the COOH-terminal tails.

Biotinylation of cell surface proteins

Primary cultures were incubated with 1.5 mg/ml sulfo NHS-SS-biotin in PBS with 1 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (PBS/MC) on ice for 12 min, then washed three times with cold PBS/MC containing 50 mM glycine. Membranes were prepared, and cell surface biotinylated proteins were precipitated with Neutravidin-agarose (Pierce Chemical Co.) and detected by Western blotting.

Immunocytochemistry

Immunocytochemistry on low density cortical or hippocampal neuron cultures ($5 \times 10^4/cm^2$) maintained as described (Craven et al., 1999) was performed at 30 DIV. EM was performed as described for DAB (Petralia and Wenthold, 1999) and immunogold (Chen et al., 2000; Sans et al., 2001). Rats were perfused with 4% PFA with or without 0.5% glutaraldehyde for immunogold or DAB, respectively. For the chart in Fig. 6, gold position was measured in 45 synapses having double labeling for pan-TARP and GluR2/3 in random areas examined in the CA1 stratum radiatum, CA3 stratum lucidum, hilus, and stratum oriens regions of the hippocampus. Double labeled synapses had at least one gold particle of each size within 150 nm on each side of the postsynaptic membrane. Animals were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85–23; NIDCD protocol no. 1022–01).

Online supplemental material

Figs. S1 and S2 are available at http://www.jcb.org/cgi/content/full/jcb.200212116/DC1. Fig. S1 shows γ-8 using a noncanonical initiator codon, and Fig. S2 shows γ-4 expressed in cultured glia.

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